The K1 Capsular Polysaccharide of *Acinetobacter baumannii* Strain 307-0294 Is a Major Virulence Factor[∇]

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Acinetobacter baumannii **is a pathogen of increasing medical importance with a propensity to be multidrug resistant, thereby making treatment challenging. Little is known of virulence traits in** *A. baumannii.* **To identify virulence factors and potential drug targets, random transposon (Tn) mutants derived from the** *A. baumannii* **strain AB307-0294 were screened to identify genes essential for growth in human ascites fluid** *in vitro,* **an inflammatory exudative fluid. These studies led to the identification of two genes that were predicted to be required for capsule polymerization and assembly. The first,** *ptk,* **encodes a putative protein tyrosine kinase (PTK), and the second,** *epsA,* **encodes a putative polysaccharide export outer membrane protein (EpsA). Monoclonal antibodies used in flow cytometric and Western analyses confirmed that these genes are required for a capsule-positive phenotype. A capsule-positive phenotype significantly optimized growth in human ascites fluid, survival in human serum, and survival in a rat soft tissue infection model. Importantly, the clearance of the capsule-minus mutants AB307.30 (***ptk*** mutant, capsule minus) and AB307.45 (***epsA*** mutant, capsule minus) was complete and durable. These data demonstrated that the K1 capsule from AB307-0294 was an important protectin. Further, these data suggested that conserved proteins, which contribute to the capsule-positive phenotype, are potential antivirulence drug targets. Therefore, the results from this study have important biologic and translational implications and, to the best of our knowledge, are the first to address the role of capsule in the pathogenesis of** *A. baumannii* **infection.**

The incidence of *Acinetobacter baumannii* infection in all venues is increasing worldwide (17, 20, 32, 34). The majority of *A. baumannii* infections have been acquired in health care facilities. *A. baumannii* has been reported to account for 1 to 3% of hospital-acquired infections, 2 to 10% of infections in intensive care units, and, from 1995 to 2002, 1.3% of nosocomial bacteremias (18, 34, 42). Both sporadic and epidemic infections occur usually after the first week of hospitalization (4, 17, 18, 25, 44). The respiratory tract, particularly in ventilated patients (6.9% of hospital-acquired pneumonias in 2003 based on NNIS data), the urinary tract, intravascular devices, surgical sites (including in postneurosurgical meningitis patients), and decubitus or diabetic ulcers are favored sites of infection (4, 25, 44). *A. baumannii* has also been described to cause infections outside the health care setting, namely, severe community-acquired pneumonia (usually in alcoholics) (2, 3, 8) and infections in U.S. service members with war-related injuries (most recently, those injured in the Iraq/Kuwait/Afghanistan regions) (7, 12, 13, 24, 39) and in survivors of the Asian tsunami in 2004 (19, 28). Mortality rates associated with *A. baumannii* infection range from 19 to 54% (20). Mortality

associated with Gram-negative bacteremia was significantly increased in patients with *A. baumannii* compared to other Gram-negative bacilli (GNB) (23, 35). The changing epidemiology, the increasing incidence of infection, and the significant mortality with *A. baumannii* establishes it as a pathogen of increasing medical importance.

The propensity for *A. baumannii* to be multidrug resistant (MDR) compounds the problems with our ability to control this emerging problem. The prevalence of MDR *A. baumannii* strains is increasing, with some exhibiting resistance to all available antimicrobials (7, 13, 22, 40, 43). Thus, treatment of infections due to *A. baumannii* has become challenging. Due to the evolution of MDR GNB, we are at risk for entering a postantibiotic era. *A. baumannii* is the "poster child" for this emerging threat (5). Further, colonization or infection with MDR *A. baumannii* was associated with an increased mortality compared to that of infection with MDR *Pseudomonas aeruginosa* (21). Unfortunately, there are virtually no new antimicrobial agents active against GNB in the pharmaceutical "pipeline." The need to understand the biology of *A. baumannii,* which may lead to logical drug design or vaccine development, is more pressing than ever.

Multiple bacterial virulence factors are required for the pathogenesis of infections caused by GNB, including *A. baumannii.* The possession of specialized virulence genes enables pathogens to infect the host efficiently. Currently, a significant gap in our knowledge of the nature of these virulence traits in *A. baumannii* exists. Surface polysaccharides, such as capsule,

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have long been recognized to be important virulence traits in GNB. However, our knowledge of the role of capsular polysaccharide in the pathogenesis of *A. baumannii* infection is nonexistent. Our group has been studying the *A. baumannii* strain AB307-0294 as a model pathogen. In order to identify virulence factors and potential drug targets, random transposon (Tn) mutants were screened to identify genes essential for growth in human ascites fluid *in vitro* and subsequently in a rat soft tissue infection model. These studies led to the identification of two genes that were predicted to be required for capsule polymerization and assembly. The first, *ptk*, encodes a putative protein tyrosine kinase (PTK), and the second, *epsA*, encodes a putative polysaccharide export outer membrane protein (EpsA) (11). In this report, we establish that these genes are required for a capsule-positive phenotype and describe a major role for capsule in the pathogenesis of *A. baumannii* infection. These data have important translational implications and support the potential of Ptk and EpsA as drug targets.

MATERIALS AND METHODS

Bacterial strains and media. *A. baumannii* strain 307-0294 (blood isolate) is an encapsulated isolate from a patient hospitalized at Erie County Medical Center, Buffalo, NY, in 1994 (38). Capsule serotypes in *A. baumannii* have not been defined. Therefore, we have designated the capsular serotype present in AB307-0294 K1. Derivatives of the wild-type (wt) strain AB307-0294 include AB307.30 (*ptk* mutant, capsule minus), AB307.30/pNLAC1 (*ptk* mutant with the pNLAC1 plasmid without an insert, capsule minus), AB307.30/pNLAC1::*ptk* (complemented *ptk* mutant, capsule positive), AB307.45 (*epsA* mutant, capsule minus), AB307.45/pNLAC1 (*epsA* mutant with the pNLAC1 plasmid without an insert, capsule minus), AB307.45/pNLAC1::*epsA* (complemented *epsA* mutant, capsule positive), AB307::Tn*17* (*lpsB* mutant, deep lipopolysaccharide [LPS] core mutation, capsule positive) (27), AB307::Tn*17*/pSS11 (complemented *lpsB* mutant, wt LPS, capsule positive) (27), and AB307::Tn*17*/pNLAC1 (*lpsB* mutant with the pNLAC1 plasmid without an insert, deep LPS core mutation, capsule positive). Strains were grown in Luria-Bertani (LB) medium unless stated otherwise and were maintained at −80°C in 50% LB broth and 50% glycerol. Ascites fluid plates were made as described previously (38). For quantitative growth curves, 100% ascites fluid was utilized.

Tn mutagenesis and mutant screening. Tn mutagenesis and screening for growth defects on ascites fluid plates was performed as described previously (38).

DNA sequencing and analysis. The locations of the Tn insertion sites in mutant derivatives of AB307-0294 were determined by chromosomal sequencing, with priming off the EZ-Tn*5* <Kan-2> Tn, as described previously (38). Sequence comparisons were performed via BLAST analysis of the nonredundant GenBank database.

Cloning of *ptk* and *epsA* and construction of complemented derivatives of AB307.30 and AB307.45. *ptk* and 336 bases upstream and 474 bases downstream were cloned via PCR-mediated amplification (forward, 5′-ATTTCAGGGCTT ATTGGTC-3′; reverse, 5′-TCATAAGCAGCAACGGCAG-3′). Likewise, *espA* and 348 bases upstream and 74 bases downstream were cloned via PCR-mediated amplification (forward, 5′-ACAAACTTCTTCTGTAGCACC-3′; reverse, 5′-AAAAATACTCTGCCATAGGG-3′). Primers contained capped SacI and AgeI sites to facilitate ligation into the vector pNLAC1 (tetracycline and ampicillin resistant). The cloned *ptk* and *epsA* genes were confirmed to be identical to those in strain AB307-0294 by bidirectional DNA sequencing. pNLAC1::*ptk* and pNLAC1::*espA* constructs were electroporated into AB307.30 and AB307.45, respectively, generating the complemented strains AB307.30/pNLAC1::*ptk* and AB307.30/pNLAC1::*espA*, respectively. In addition, pNLAC1 without the insert was electroporated into AB307.30 and AB307.45 to generate the control strains AB307.30/pNLAC1 and AB307.45/pNLAC1, respectively.

Generation of the MAb 13D6, which is specific for the K1 capsule expressed in AB307-0294. Monoclonal antibodies (MAbs) were developed and screened as described previously (6, 26). MAbs that reacted to proteinase K-resistant, periodate-sensitive bacterial surface epitopes were selected for further analyses. The MAb 13D6 met these criteria and was shown to be directed against the K1 capsule (see "Confirmation that AB307.30 and AB307.45 are capsule-deficient derivatives of AB307-0294" below).

Flow cytometry. Flow-cytometric assessment of antibody binding was performed as described previously (36). In brief, the appropriate strains were grown in LB medium, diluted to 1×10^6 CFU, washed in 100 μl of $1\times$ phosphate-buffered saline (PBS) (pH 7.2), resuspended in 100 μl of 1:100-diluted hybridoma supernatants (complement inactivated), and incubated while rotating for 60 min at 37°C. Preliminary experiments established that this incubation time and dilution resulted in maximal antibody binding. Postincubation, the bacteria were washed once with 0.5 ml of $1\times$ PBS, resuspended in 100 μl of goat anti-mouse IgG-IgM fluorescein isothiocyanate (FITC)-labeled conjugate (Caltag Laboratories, Burlingame, CA), and incubated while rotating at 37°C for 30 min. The labeled bacteria were washed, resuspended in 500 μl of $1\times$ PBS, and transferred to 5-ml flow cytometry tubes (Falcon 352054). Antibody binding was analyzed by flow cytometry. Ten thousand bacterial events were gated in the forward-scatter versus side-scatter plot, discriminating them from "debris," and antibody-bound-bacterial events were assessed in the FL-1 channel and are reported as percentages of positive cells or geometric mean fluorescence (GMF). Binding in the absence of antibody (PBS alone) served as the autofluorescence control, and binding with the FITC-labeled conjugate alone served as the nonspecific binding control, to which experimental samples were compared.

Phenotypic analyses of *A. baumannii* K1 capsule and LPS. LPS and capsular polysaccharide preparations, obtained by treating whole-cell lysates with proteinase K, were resolved by SDS-16% polyacrylamide gel electrophoresis and subjected to Western blot analyses with MAbs 13D6 and 13C11, as described previously (27).

Rat soft tissue infection model. The rat soft tissue infection model, approved by the University at Buffalo and Veterans Administration Institutional Animal Care Committee, was used as reported previously (37). The mean starting inocula for AB307-0294, AB307.30, and AB307.45 were 3×10^5, 2.5×10^5, and 1.1×10^5 CFU/ml, respectively.

Serum bactericidal assay. Complement-mediated bactericidal assays were performed as described previously (37, 38).

Statistical analyses. Data are presented as means ± standard errors of the mean (SEM). *P* values of $0.05/n$ (where *n* is the number of comparisons) are considered statistically significant based on the Bonferroni correction for multiple comparisons, and *P* values of $>0.05/n$ but <0.05 are considered as representing a trend. To normalize *in vitro* and *in vivo* data, $\log_{10}$-transformed values were utilized, the area under each curve was calculated, and these areas were compared using two-tailed unpaired *t* tests (Prism 4 for Macintosh; GraphPad Software Inc.).

RESULTS

Identification of AB307.30 (*ptk* mutant, capsule minus) and AB307.45 (*espA* mutant, capsule minus). To identify factors in *A. baumannii* that are necessary for growth and survival in human infection, we screened random Tn mutants for growth on ascites fluid plates, an *ex vivo*-modified minimal medium representing a common environment for extracellular bacterial pathogens such as *A. baumannii* (38). This screen resulted in the identification of AB307.30 and AB307.45. Quantitative growth assays confirmed that these mutants exhibited defective growth in human ascites fluid compared to that of AB307-0294 (wt, capsule positive) (see "Capsule is a critical protectin requisite for optimal growth and survival in human ascites fluid and survival against complement-mediated bactericidal activity *in vitro*" below).

Chromosomal sequencing determined that the Tn insertion within AB307.30 was in *ptk* (locus tag ABBFA003461, between nucleotides [nt] 3688640 and 3688641) and within AB307.45 was in *epsA* (locus tag ABBFA003459, between nt 3687340 and 3687341) (1) (Fig. 1). BLAST analysis of the deduced protein sequence generated from these open reading frames identified Wcz as the *Escherichia coli* ortholog of PTK and Wza as the *E. coli* ortholog of EpsA (11). Both of these genes encode proteins that facilitate the polymerization and transport of capsular polysaccharide. PTK and EpsA consist of 727 amino acids (aa) and 333 aa, have predicted molecular masses of 81,639
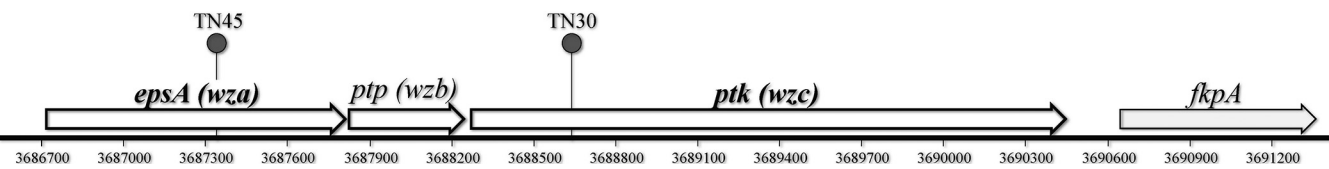
FIG. 1. Genetic organization of an *A. baumannii* capsule cluster containing polymerization and transport genes. Eps I polysaccharide export outer membrane protein (*epsA*; locus tag ABBFA003459; *E. coli* ortholog, *wza*). Low-molecular-weight protein tyrosine phosphatase (*ptp*; locus tag ABBFA003460; *E. coli* ortholog, *wzb*). Protein tyrosine kinase (*ptk*; locus tag ABBFA003461; *E. coli* ortholog *wzc*). An FK506-binding protein-type peptidyl-prolyl *cis-trans* isomerase (*fkpA*) is outside the capsule gene cluster. Ruler numbering indicates the genomic coordinates (NCBI accession number CP001172) (1).

kDa and 40,512 kDa, and have pIs of 6.39 and 6.91, respectively. Reverse transcription-PCR (RT-PCR) analyses confirmed that *ptk* and *epsA* were not cotranscribed with other genes (data not shown). Nonetheless, to establish that the Tn insertion within these genes did not result in a polar effect, complementation studies were performed and were confirmatory.

Confirmation that AB307.30 and AB307.45 are capsule-deficient derivatives of AB307-0294. To confirm that both mutants were capsule minus, flow cytometry studies were performed comparing the reactivity of MAb 13D6 on live AB307-0294 to its reactivities on AB307.30, AB307.45, and their re-

spective complemented derivatives, AB307.30/pNLAC1::*ptk* and AB307.45/pNLAC1::*epsA* (Fig. 2; Table 1). As predicted, MAb 13D6 bound AB307-0294 and the complemented mutants AB307.30/pNLAC1::*ptk* and AB307.45/pNLAC1::*espA* but not AB307.30 or AB307.45. Antibody binding was nearly restored to the degree observed for the wild type for AB307.45/pNLAC1::*espA*, but complementation did not completely restore antibody binding for AB307.30/pNLAC1::*ptk*. To further confirm that AB307.30 and AB307.45 were defective in capsule expression and not lipopolysaccharide (LPS), we performed additional studies with the recently described MAb 13C11 and LPS core mutant AB307::Tn*17* (27). MAb
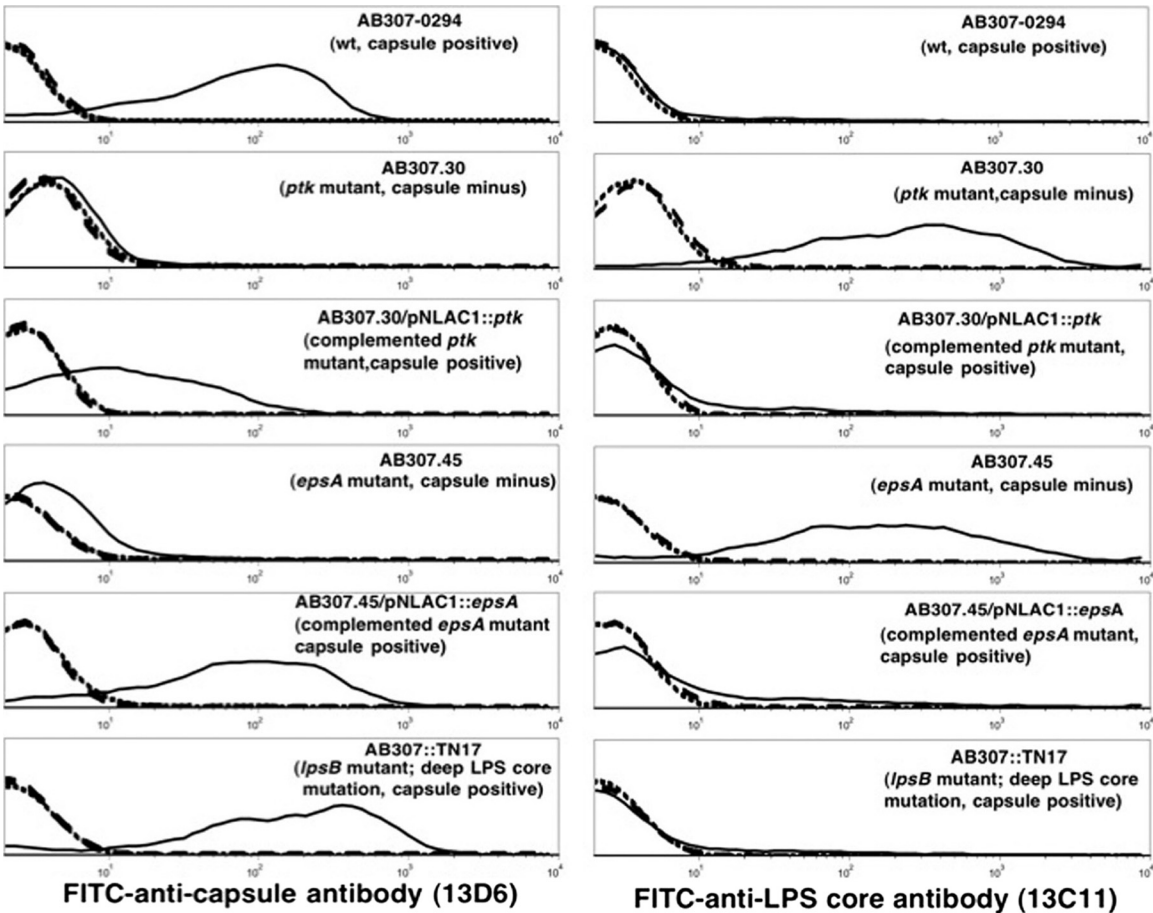

FIG. 2. Confirmation that MAb 13D6 recognizes the K1 capsular serotype by flow cytometry. Binding of MAb 13D6 (left) and MAb 13C11 (right) to the indicated strains. MAB 13C11 recognizes a deep LPS core epitope. Small dashed back line, PBS control; large dashed gray line, FITC control; solid line, bacteria.

TABLE 1. Percentages of bacterial cells bound by MAb 13C11 (anti-LPS core) and 13D6 (anti-K1 capsule) as assessed by flow cytometry

Exptl parameter	% of cells bound[a]					
	AB307	AB307.30	AB307.30/ pNLAC1::ptk	AB307.45	AB307.45/ pNLAC1::epsA	AB307::Tn17
PBS (autofluorescence control)	0.2 ± 0.08	1.8 ± 0.8	0.6 ± 0.2	0.9 ± 0.2	0.5 ± 0.1	0.4 ± 0.07
FITC alone (conjugate control)	1.1 ± 0.3	2.7 ± 0.5	1.6 ± 0.2	3.3 ± 0.2	2.0 ± 0.4	2.1 ± 0.3
13C11 (anti-LPS core)	$7.3 \pm 0.2*$	$87.1 \pm 1.1*$	$10.4 \pm 1.1**$	$86.0 \pm 1.8*$	$21.7 \pm 1.4*$	$11.4 \pm 1.3***$
13D6 (anti-K1 capsule)	$86.8 \pm 0.8*$	4.0 ± 0.1	$44.6 \pm 3.6*$	$11.3 \pm 0.8****$	$73.3 \pm 1.5*$	$88.0 \pm 0.6*$

[a] AB307 (wt, capsule positive), AB307.30 (*ptk* mutant, capsule minus), AB307.30/pNLAC1::*ptk* (complemented *ptk* mutant, capsule positive), AB307.45 (*epsA* mutant, capsule minus), AB307.45/pNLAC1::*epsA* (complemented *epsA* mutant, capsule positive), AB307::Tn*17* (*lpsB* mutant, deep LPS core mutation, capsule positive). Positivity is relative to that of the FITC control. *, $P < 0.0001$; **, $P = 0.0009$; ***, $P = 0.0010$; ****, $P = 0.0002$; compared to bacteria stained with FITC conjugate alone. Values are means $\pm$ SEM ($n = 6$).

13C11 reacts to an LPS core epitope expressed on the surface of AB307-0294 (27). Figure 2 shows that MAb 13D6 reacts to AB307::Tn*17* but that MAb 13C11 does not react to this LPS mutant, which excludes the possibility that MAb 13D6 recognized the O-antigen moiety of LPS. Further, MAb 13C11 bound AB307.30 and AB307.45, as predicted, but did not react to AB307-0294, AB307.30/pNLAC1::*ptk*, or AB307.45/pNLAC1::*espA*, presumably due to the steric hindrance of capsule (Fig. 2, right, and Table 1).

To further confirm that AB307.30 and AB307.45 were unable to express capsule, proteinase K-treated whole-cell lysates were subjected to Western analysis using MAb 13D6 and MAb 13C11 (Fig. 3). MAb 13D6-probed immunoblots indicated that this antibody reacted to the high-molecular-mass capsular polysaccharide produced by AB307-0294 and the LPS-deficient mutant AB307::Tn*17* but exhibited no reactivity to AB307.30 or AB307.45 (Fig. 3A). MAb 13D6 reactivity was restored by in *trans* complementation for both Tn mutants. Corresponding immunoblots were probed with MAb 13C11,
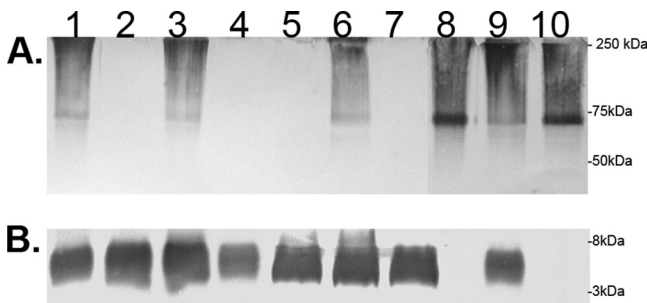

FIG. 3. Confirmation that AB307.30 and AB307.45 are capsule deficient by Western analysis. Composite Western blotting was performed on proteinase K-treated whole-cell lysates; the lysates were then probed with MAb 13D6, which recognizes the capsular polysaccharide epitope, and MAb 13C11, which recognizes a deep LPS core epitope. Binding of MAb 13D6 (A) and of MAb 13C11 (B) to AB307-0294 (lane 1), AB307.30 (lane 2), AB307.30/pNLAC1::*ptk* (lane 3), AB307.30/pNLAC1 (*ptk* mutant with the pNLAC1 plasmid without the insert, capsule minus) (lane 4), AB307.45 (lane 5), AB307.45/pNLAC1::*epsA* (lane 6), AB307.45/pNLAC1 (*epsA* mutant with the pNLAC1 plasmid without the insert, capsule minus) (lane 7), AB307::Tn*17* (lane 8), AB307::Tn*17*/pSS11 (complemented *lpsB* mutant, wt LPS, capsule positive) (lane 9), and AB307::Tn*17*/pNLAC1 (*lpsB* mutant with the pNLAC1 plasmid without the insert, deep LPS core mutation, capsule positive) (lane 10). The positions of molecular mass standards are shown to the right.

which reacts only to wild-type LPS (Fig. 3B), and all strains except AB307::Tn*17* were reactive as expected. Taken together, these data demonstrate that the loss of functional PTK and EpsA activities specifically affected the production of capsular polysaccharide by AB307-0294 and did not alter the production of LPS. The fact that AB307::Tn*17*, which produces a deeply truncated LPS glycoform consisting only of KDO_2-lipid A (27), maintains MAb 13D6 reactivity further demonstrated that the AB307-0294 capsular polysaccharide is distinct from the LPS molecule produced by this strain. Moreover, these data confirm that the poor binding of MAb 13C11 observed in the flow cytometric studies was due to steric hindrance (29, 41).

Taken together, these data demonstrate that MAb 13D6 recognizes the K1 capsule. Further, these data also establish that AB307.30 and AB307.45 are defective in capsule expression. Lastly, these data confirm that PTK and EpsA are essential for a capsule-positive phenotype but do not contribute to LPS synthesis.

Capsule is a critical protectin requisite for optimal growth and survival in human ascites fluid and survival against complement-mediated bactericidal activity *in vitro*. It was important to confirm that the observed growth difference of AB307.30 and AB307.45 on ascites fluid plates was not due to an innate growth defect. Therefore, the ability of AB307-0294 and those of its mutant derivatives AB307.30 and AB307.45 to grow in laboratory medium (Luria-Bertani broth) were assessed and found to be similar ($P = 0.13$ and $P = 0.26$ for AB307.30 and AB307.45, respectively, compared to AB307-0294) (Fig. 4).

Next, comparative quantitative growth assays in human ascites fluid were performed. As expected, AB307-0294, AB307.30/pNLAC1::*ptk*, and AB307.45/pNLAC1::*epsA* grew in ascites fluid, whereas the overall growth and survival of AB307.30, AB307.30/pNLAC1 (*ptk* mutant with the pNLAC1 plasmid without the insert, capsule minus), AB307.45, and AB307.45/pNLAC1 (*espA* mutant with the pNLAC1 plasmid without the insert, capsule minus) were significantly less ($P < 0.0001$, $P < 0.0001$, $P = 0.0002$, and $P = 0.0005$, respectively, compared to AB307-0294) (Fig. 5A and B). These data demonstrated that a capsule-positive phenotype was necessary for optimal growth and survival in this *ex vivo* human body fluid. Importantly, the complemented mutants confirmed that PTK and EpsA are essential for optimal growth in ascites fluid.

Ascites fluid is known to contain active complement; therefore, we studied the effect of human serum on AB307-
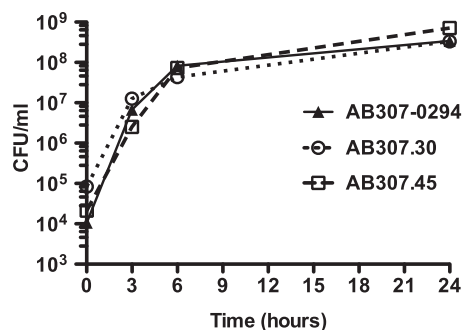
FIG. 4. The levels of growth of AB307-0294 (wt), AB307.30 (*ptk* mutant, capsule minus), and AB307.45 (*epsA* mutant, capsule minus) in laboratory medium were similar. The levels of growth of AB307-0294 ($n = 3$), AB307.30 ($n = 3$), and AB307.45 ($n = 3$) were assessed at 0, 3, 6, and 24 h in Luria-Bertani medium and were similar ($P = 0.13$ and $P = 0.26$ for AB307.30 and AB307.45 compared to AB307-0294, respectively) (two-tailed unpaired *t* test). Data are means ± SEM.

0294 compared to that on the Tn mutants AB307.30 and AB307.45 and their complemented derivatives. AB307-0294 and AB307.30/pNLAC1::*ptk* were resistant to the bactericidal activity of 90% human serum (Fig. 6A). In contrast,
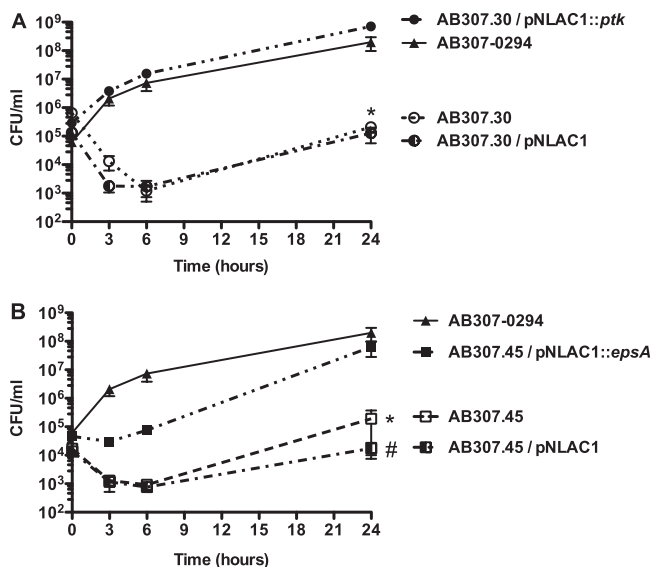

FIG. 5. AB307.30 (*ptk* mutant, capsule minus) and AB307.45 (*epsA* mutant, capsule minus) were significantly killed in human ascites fluid compared to their wild-type parent AB307-0294. (A) The growth of AB307-0294 ($n = 6$), AB307.30 ($n = 6$), AB307.30/pNLAC1 (*ptk* mutant with the pNLAC1 plasmid without the insert, capsule minus) ($n = 5$), and AB307.30/pNLAC1::*ptk* ($n = 4$) was assessed at 0, 3, 6, and 24 h. Data are means ± SEM. The survival of AB307.30 and AB307.30/pNLAC1 was significantly decreased in ascites fluid compared to that of AB307-0294 (∗, $P < 0.0001$, two-tailed unpaired *t* test). The levels of growth of the wild-type strain AB307-0294 and the complemented *ptk* mutant AB307.30/pNLAC1::*ptk* were similar ($P = 0.61$). (B) The growth of AB307-0294 ($n = 6$), AB307.45 ($n = 4$), AB307.45/pNLAC1 (*epsA* mutant with the pNLAC1 plasmid without the insert, capsule minus) ($n = 4$), and AB307.45/pNLAC1::*epsA* ($n = 4$) was assessed at 0, 3, 6, and 24 h. The survival of AB307.45 and AB307.45/pNLAC1 was significantly decreased in ascites fluid compared to that of AB307-0294 (∗, $P = 0.0002$ for AB307.45; #, $P = 0.0005$ for AB307.45/pNLAC1). The levels of growth of the wild-type strain AB307-0294 and the complemented *epsA* mutant AB307.45/pNLAC1::*epsA* were similar ($P = 0.09$).
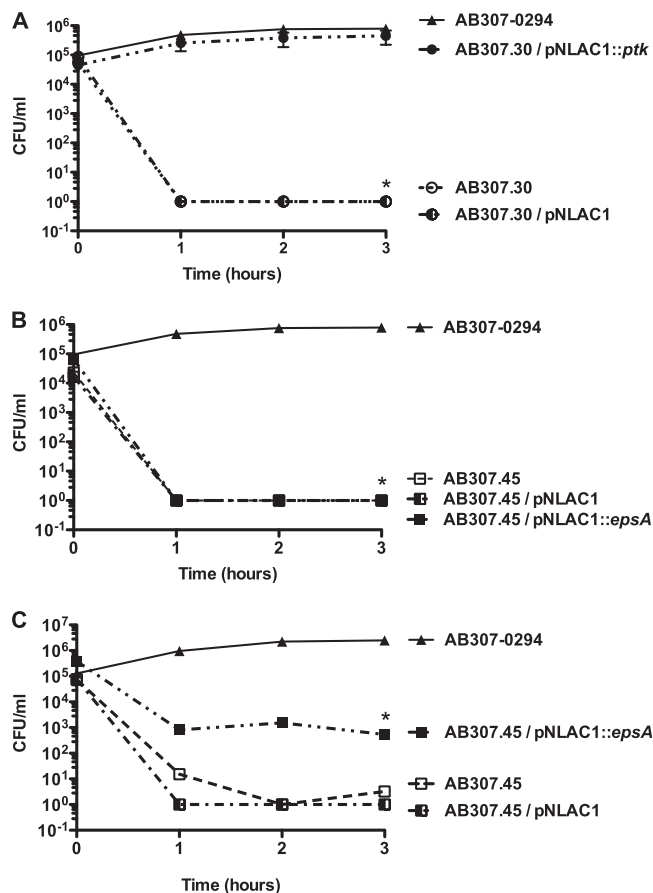

FIG. 6. AB307.30 (*ptk* mutant, capsule minus) and AB307.45 (*epsA* mutant, capsule minus) were significantly killed in human serum compared to their wild-type parent, AB307-0294 (capsule positive). (A) The survival in 90% human serum of AB307-0294 ($n = 6$), AB307.30 ($n = 8$), AB307.30/pNLAC1 ($n = 4$), and AB307.30/pNLAC1::*ptk* ($n = 4$) was assessed at 0, 1, 2, and 3 h. AB307.30 and AB307.30/pNLAC1 were significantly killed in serum compared to AB307-0294 (∗, $P < 0.0001$; two-tailed unpaired *t* test). The rates of survival of wild-type strain AB307-0294 and the complemented *ptk* mutant AB307.30/pNLAC1::*ptk* were similar ($P = 0.1$). (B) The survival in 90% human serum of AB307-0294 ($n = 6$), AB307.45 ($n = 4$), AB307.45/pNLAC1 ($n = 4$), and AB307.45/pNLAC1::*epsA* ($n = 4$) was assessed at 0, 1, 2, and 3 h. AB307.45, AB307.45/pNLAC1, and AB307.45/pNLAC1::*epsA* were significantly killed in serum compared to AB307-0294 (∗, $P < 0.0001$; two-tailed unpaired *t* test). (C) The survival in 20% human serum of AB307-0294 ($n = 4$) AB307.45 ($n = 4$), AB307.45/pNLAC1 ($n = 4$), and AB307.45/pNLAC1::*epsA* ($n = 4$) was assessed at 0, 1, 2, and 3 h. AB307.45 and AB307.45/pNLAC1 were significantly killed in serum compared to AB307-0294 (∗, $P < 0.0001$; two-tailed unpaired *t* test). The survival of the complemented *epsA* mutant AB307.45/pNLAC1::*epsA* was significantly greater than that of the noncomplemented mutants AB307.45 and AB307.45/pNLAC1 ($P = 0.0007$ and 0.0002, respectively) but still significantly less than that of the wild-type strain, AB307-0294 ($P = 0.0002$). All strains were also assessed in the presence of 90% heat-inactivated (56°C for 30 min) normal human serum, and their levels of growth were similar (data are not shown).

AB307.30, AB307.30/pNLAC1, AB307.45, and AB307.45/pNLAC1 were dramatically sensitive to 90% human serum, undergoing a rapid (<1 h), nearly 5-log kill ($P < 0.0001$, compared to AB307-0294 and AB307.30/pNLAC1::*ptk*) (Fig. 6A and B). Interestingly, complementation of AB307.45 with pNLAC1::*espA* did not restore resistance to 90% human serum
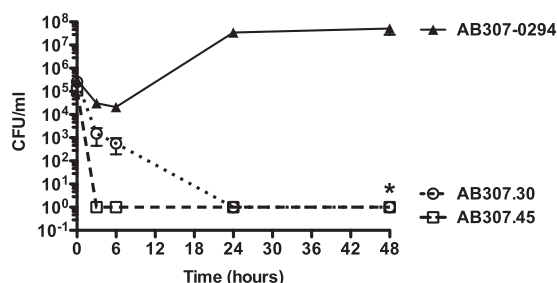
FIG. 7. AB307.30 and AN307.45 were significantly killed in the rat soft tissue infection model compared to their wild-type parent, AB307-0294. Rats were challenged with AB307-0294 (4 to 6 rats per time point), AB307.30 (4 rats), and AB307.45 (7 rats). Bacterial titers were determined at 0, 3, 6, 24, and 48 h. Data are means ± SEM. The survival of AB307.30 and AB307.45 was significantly decreased compared to that of AB307-0294 (*, $P < 0.0001$; two-tailed unpaired t test).

but did restore partial resistance to 20% human serum (Fig. 6C). These data further confirm the importance of the capsule as an *A. baumannii* virulence factor and of PTK and EpsA as essential proteins for serum survival.

Capsule is a critical protectin *in vivo*. A validation of our *in vitro* findings was needed to confirm that capsule, and therefore PTK and EpsA, were essential for survival *in vivo*. This was accomplished by comparing the levels of growth and survival of AB307-0294, AB307.30, and AB307.45 in an established rat soft tissue infection model. This model is particularly clinically relevant since *A. baumannii* has been increasingly recognized as a cause of a variety of soft tissue infections (7, 12). Further, it has been validated for studying *A. baumannii* by our group (37). Compared to AB307-0294, AB307.30 and AB307.45 demonstrated a highly significant decrease in survival in this model ($P < 0.0001$) (Fig. 7). These data clearly demonstrated that capsule is critical for the survival of AB307-0294 *in vivo* and therefore that PTK and EpsA are essential in this setting.

DISCUSSION

Although recent reports have described the production of two surface polysaccharides produced by *A. baumannii*, the extracellular polysaccharide poly-beta-(1-6)-N-acetylglucosamine (9) and LPS (27), remarkably, almost nothing is known about any aspect of the capsular polysaccharides produced by this bacterial pathogen. The data presented in this report begin to fill in this critical knowledge gap. First, it was clearly demonstrated that the K1 capsule from the *A. baumannii* strain AB307-0294 was necessary for optimal growth in human ascites fluid (Fig. 5) and survival in human serum (Fig. 6), both of which contain complement, a critical host defense system that is instrumental in protecting against extracellular bacterial pathogens such as *A. baumannii*. Further, capsule was requisite for survival in a rat soft tissue infection model (Fig. 7). Most importantly, the magnitude and durability of the clearance of the capsule-minus mutants AB307.30 and AB307.45 was remarkable (Fig. 7). These data clearly established capsule as a critical protectin for *A. baumannii*.

Our strategy to identify genes important for the growth and survival of *A. baumannii* by screening Tn mutants on human ascites fluid plates is an innovative modification of previously established methods. Ascites fluid plates represent an *ex vivo* screening tool reflective of inflammatory extracellular fluid and therefore mimic the host environment that occurs with infection. This simple innovation was critical for the efficiency of our approach. By selecting for Tn mutants on laboratory medium and then screening for decreased or absent growth on ascites fluid plates, we were able to identify genes that are both requisite and expressed *in vivo* in one step. This approach is advantageous in that it is unbiased and highly efficient. We do not select the genes but allow the genetic screen, designed to identify the phenotype of *in vivo* essentiality, to dictate the choices. This genetic approach also avoids a potential problem that may occur when using *in silico*, genomic, or proteomic approaches. Genes/proteins predicted to possess biologic significance by these methods might subsequently prove not to have a phenotype of interest, often after the cumbersome and time-consuming process of construct generation and testing.

Although the mechanism by which capsule confers this survival advantage has not yet been established *in vivo*, given the increased serum sensitivity of AB307.30 and AB307.45 *in vitro*, it is logical to infer that resistance to the bactericidal activity of complement is one means. Whether capsule also serves to protect against the effects of professional phagocytes (e.g., neutrophils and macrophages) or antimicrobial peptides remains to be tested. To date, we have not performed any studies on the mechanisms by which PTK and EpsA facilitate capsule expression. However, a substantial body of work has been done on their *E. coli* orthologs, Wcz and Wza, respectively. These proteins are part of a multiprotein complex that spans the cell membrane. Although the precise roles of the participating proteins in this process are still being defined, Wcz is an inner membrane tyrosine kinase that regulates capsule assembly and transport and Wza is an outer membrane protein that, in a complex with Wza, enables the transport of capsule across the periplasm and outer membrane (10, 11, 14). In *Acinetobacter lwoffii* and *Acinetobacter johnsonii*, the *ptk* ortholog *wzc* has also been shown to possess autophosphorylating tyrosine kinase activity (15, 16, 30), and in *A. lwoffii*, it has been demonstrated that it is required for optimal expression of the extracellular galactosamine-containing lipoheteropolysaccharide bioemulsifier (30).

One of the promises of the field of microbial pathogenesis was to identify potential drug targets. A strategy to inactivate such targets is supported by recent results (31, 33), thereby validating the potential of virulence factors or their regulators as drug targets. PTK and EpsA possess many of the characteristics requisite for an anti-virulence drug target. The loss of PTK or EpsA resulted in a complete and durable ≥5-log kill of AB307-0294 *in vivo*, fulfilling an important criterion for a potential antivirulence drug target. Next, and importantly, PTK and EpsA do not have any significant homology to human proteins (data not shown). PTK and EpsA were 100% prevalent in the 8 *A. baumannii* strains for which sequence is available: 6 *A. baumannii* genomes in the public domain (*A. baumannii* strains ATCC 17978, ATCC 0057, AYE, ACICU, SDF, and ATCC 19606) and 2 *A. baumannii* strains sequenced by our group in addition to AB307-0294, namely, AB853 (blood isolate from Iraq) and AB979 (environmental isolate from Iraq) (unpublished data). The putative protein sequences of

PTK of AB307-0294 and EpsA of AB307-0294 were compared to the sequences in these *A. baumannii* strains and were found to have 91 to 100% identities and 96 to 100% similarities. These data suggest that PTK and EpsA are potential antimicrobial targets.

In summary, we have demonstrated the importance of the K1 capsule from the *A. baumannii* strain AB307-0294 as a protectin *in vitro* and *in vivo*. These results have important biologic and translational implications.

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